Synopsis of the thesis

Studies on tissue culture and transformation of selected genotypes of *Sorghum bicolor* (L.) Moench

By

SEERAM NAGESWARA RAO M.Sc.

Under the Guidance of

Prof. N. SARADA MANI (GUIDE)
&
DR. P. K. RATNA KUMAR (CO-GUIDE)
Department of Botany
Andhra University
Visakhapatnam

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Sorghum (*Sorghum bicolor* (L.) Moench) belongs to family poaceae. It is a wonder crop from physiological point of view. It is the fifth most important crop after rice, wheat, maize and barley. It is a cereal crop of world wide importance and has the unique ability to grow under a wide array of harsh environmental conditions. It is mainly grown in arid and semi-arid zones of the world. It is the staple food of tens of millions of poor people in the semi-arid tropics. However, it has not received adequate attention from crop biotechnology industry. The improvement in grain production achieved so far in India has been mainly through classical breeding methods. There are several biotic and abiotic stresses that affect the productivity of this crop, some of which are too complex to tackle using only traditional approaches. *Sorghum* crop yield can be improved further in relatively short periods and in a more economic way by a judicious combination of conventional breeding, and the novel biotechnology methodologies. The current advantages in biotechnological tools such as *in vitro* cell culture and genetic engineering need to be employed to enhance crop yield.

An efficient and reproducible regeneration system is a prerequisite for applying *in vitro* techniques in genetic transformation studies. Therefore, a fundamental understanding of the *in vitro* system is necessary to undertake transformation studies. Also genetic engineering in *Sorghum* has not been possible for a long time because of fundamental problems associated with the method of gene transfer into target tissue and their subsequent regeneration. *In vitro* culturing of *Sorghum* is very much influenced by genotype, explant, media composition and hormones. Every genotype requires a special nutrient composition, cultural conditions and environmental factors for efficient regeneration. A critical point in developing an efficient transformation protocol is to find

the right combination of these many factors that act together during transformation. The regeneration of fertile plants following genetic transformation is an added problem. In addition to the regeneration protocols and the system of DNA delivery, optimization of the factors for gene transfer, selection and post transformation regeneration are important factors in order to increase the efficiency of *Sorghum* production.

The present work was undertaken with an objective to develop an efficient *in vitro* plant regeneration protocols from various explants of *Sorghum* i.e., immature embryo, mature embryo, immature inflorescence and shoot meristem segments for the use of efficient genetic transformation studies. Though several authors have reported callus induction and regeneration from different explants of *Sorghum*, yet several aspects like multiple shoot induction, direct somatic embryogenesis etc. remains unexplored. Therefore, the present work was contemplated with the following objectives:

- To develop an efficient *in vitro* plant regeneration protocols from various explants of *Sorghum* i.e., immature embryo, mature embryo, immature inflorescence and shoot meristem.
- To standardize optimal conditions for multiple shoot induction to obtain large number of regenerated plants.
- To standardize suitable *in vitro* conditions for direct somatic embryogenesis to reduce the time required for the production of regenerated plants.
- To study the pathway of somatic embryogenesis by using Scanning Electron Microscopy (SEM).

- To study different genotypes of *Sorghum* for their ease and efficiency of regeneration and genetic transformation by *Agrobacterium* mediated method of gene transfer.

Callus induction and regeneration studies of different explants of *Sorghum*

Six varieties of *Sorghum* (IS 3040, IS 3949, IS 81, IS 8191, IS 2746 and IS 8887) were selected among 30 varieties based on callus induction, regeneration response, growth rate of callus and their yielding ability in order to differentiate different explants.

For callus initiation, healthy immature embryos, mature embryos, immature inflorescences and shoot meristems were used as explants. Callus initiation occurred after inoculation of explants on MS medium supplemented with different concentrations and combinations of auxins and cytokinins. Cultures were maintained in the dark at 25 ± 2^0C and subcultured at intervals of 21 days. At the end of first subculture, those cultures containing smooth, shiny, globular structures were defined as embryogenic callus and those having unorganized, creamy or yellow coloured structures as non-embryogenic callus. A combination of 2.0 mg/L 2,4-D + 0.5 mg/L Kinetin for immature embryos, 1.5 mg/L 2,4-D + 0.5 mg/L Kinetin for immature inflorescence, 2.0 mg/L 2,4,5-T + 1.0 mg/L Zeatin and 2.5 mg/L 2,4-D + 1.5 mg/L BAP for shoot meristems promoted efficient callusing instead of taking alone cytokinins or auxins. For regeneration, MS medium supplemented with 1-2 mg/L of BAP/KN/TDZ and rooting medium supplemented with NAA at 1.0 mg/L was used. Root initiation occurred within 9-12 days of inoculation. In this chapter an efficient protocol for tissue culture was established.

Multiple shoot and root induction from different explants

Multiple shoot induction will be helpful to obtain large number of regenerated plants in *in vitro* culture. Induction of multiple shoots and roots was achieved by using the embryogenic calli from the immature embryo, mature embryo, immature inflorescence and shoot meristem explants of all the six varieties. The white embryogenic callus converted into green colour shoot buds after transferring on to regeneration medium containing different Plant growth regulators at 1.0 mg/L to 3.0 mg/L concentrations. Efficient shoot response was observed on 1.5 mg/L from immature embryo, 2.0 mg/L Zn from mature embryo, 2.0 mg/L from immature inflorescence and 1.5 mg/L BAP from shoot meristem. Shoot buds were proliferating to shoots within 6-12 days. The amount of cytokinins used for plant regeneration appeared to be critical in producing a good number of shoots. Regeneration can be further enhanced by exposing the organized callus to combination of cytokinins and auxins. The combination of BAP, TDZ and IAA along with L-asparagine, L-proline and Casein hydrolysate was more favourable for multiple shoot induction. Maximum number (106) of multiple shoots was observed in IS 3040. Multiple root induction was achieved by using MS medium supplemented with 1.0 mg/L of NAA. Root initiation started within 9-12 days. Multiple root number depended on shoot number. A maximum number of roots 220 was observed in the variety IS 3040. Significant differences occurred between the genotypes in the induction of multiple shoots and roots. Conditions for elongation of shoots, *in vitro* rooting and hardening of plants were standardized.

Direct Somatic embryogenesis from different explants of *Sorghum*

Plant regeneration via either direct shoot organogenesis or direct somatic embryogenesis from explants is a prerequisite for genetic transformation. The most frequent approach for cereal regeneration includes initiation of embryogenic calli from immature tissues. Unfortunately, intervening callus phase is disadvantages because of the possible somaclonal variation. In this chapter we focused on regeneration via direct somatic embryogenesis from immature embryo, mature embryo, immature inflorescence and shoot meristem of six *Sorghum* genotypes. Direct somatic embryogenesis offers several advantages in crop improvement i.e., large scale clonal propagation, somatic seed production and development of artificial seeds etc. In the present study, shorter time required to obtain direct somatic embryogenesis might contribute to minimize somaclonal variation. The high sensitivity of explants with TDZ in combination with KN and ZN along with the concentration of $AgNO_3$, casein hydrolysate, L-proline and L-asparagine, temperature, light and other media elements have permitted the programming of direct somatic embryogenesis in diverse explants of *Sorghum bicolor* (L.) Moench. Maximum number of somatic embryo frequency was achieved in the variety IS 3040.

Somatic embryogenesis pathway from immature inflorescence derived calli using Scanning electron microscopy (SEM)

The immature inflorescence segments of *Sorghum* gave good response for induction and development of somatic embryos. Callus induction and subsequent plant regeneration from this explant derived calli through somatic embryogenic pathway was elucidated.

Histology and development of somatic embryo initiation, formation, development and regeneration were described by using SEM and light microscopy. Three weeks after culturing, these cells got differentiated into medium sized, highly cytoplasmic embryogenic cells. These cells were easily distinguishable from the proactive epidermal layer due to their greater size and dense cytoplasm. After 4 weeks of culture, these richly dividing cells occupied several layers immediately after the epidermis, followed by small, large and medium sized cells with prominent nucleus, representing different stages of differentiation. Internal segmenting divisions in many of these cells gave rise to discrete groups of cells on the callus surface. Each group was distinct and separate from each other cell by thick wall. A rapid localized proliferation occurred in meristematic activity zone, producing a lobed callus showing the absence of vascular connection with the parent tissue. The development of vascular initials in the lobed callus revealed features of early embryogenic callus development. This lobed structure is embryogenic tissue containing several layers of embryogenic cells. Two types of calli were distinguished. The embryogenic callus is mostly compact, organized, and white in colour, and contains large number of small, richly cytoplasmic, starch containing meristematic cells. The non-embryogenic callus is soft, unorganized, and contains sparsely cytoplasmic, vacuolated, and large cells devoid of any prominent metabolic reserves.

Production of transgenic plants by *Agrobacterium*-mediated genetic transformation

Agrobacterium-mediated method of transformation is feasible in *Sorghum*, as evidenced by transient GUS expression. There was variation in the GUS expression depending on the explants used for transformation. Transformants were obtained from the

three explants of the genotype IS 3040 used in this study and thus the above-developed protocol can be used for *Sorghum* transformation to introduce other agronomically desirable traits such as downy mildew resistance and /or nutritional quality improvement.

Finally, the present study has also succeeded in producing transgenic *Sorghum* plantlets from the immature inflorescence, immature embryos and shoot meristem explants of the genotype, IS 3040 out of six genotypes (IS 3040, IS 3949, IS 8191, IS 81, IS 2746 and IS 8887) by using *Agrobacterium* strain LBA 4404 with pCAMBIA 1305.1 (for GUS expression). The transformation protocol described recovered transformants within 15-20 weeks after culture initiation. Though transformation is successful, it is well established that a number of known and unknown factors reduce the efficiency of the process. Therefore, further studies should include introduction of the gene/s of interest into the best responding genotype, IS 3040, of *Sorghum* and subsequently, the number of integrated sequences, transgene expression, etc., should be determined in the transformed and regenerated plants.